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Hydrogen-Bonded Complexes of Adenine and Thymine Nucleoside Alkyl Phosphotriesters in Deuteriochloroform†

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ABSTRACT: The association of neutral analogs of adenine and thymine dinucleoside monophosphates in deuteriochloroform was investigated by infrared spectroscopy. This is the first time that such studies have been done at the dimer level; because of solubility requirements, such experiments could be done only when nucleoside alkyl phosphotriesters became available. Association constants for both self-association and cross-association were determined by analysis of divergence from Beer's law of NH and NH_2 stretching bands. The complementary dimers associated with an intrinsic constant of 192 M^{-1} as compared to a value of 92 M^{-1} for the corresponding monomers. The relatively low value for the dimers indicates that the association process is not cooperative. The two residues of the dimers behave nearly independently in the association process; therefore, the restriction on the rotation about the bonds of the backbone must be small. This conclusion is discussed in relation to recent reports on unperturbed dimensions of polynucleotides in aqueous solution. The results reinforce the conclusion from the studies on polynucleotides that the restriction of the rotation of the backbone of a polynucleotide in solution cannot be due to steric hindrance alone. Our data support the suggestion that the electrostatic interaction between the charged groups of the polynucleotide backbone and that between the charged groups and water could be the cause of the rigidity of the polynucleotide in a random coil conformation observed in aqueous solution.

Hydrogen bonding of purine and pyrimidine bases and nucleosides in organic solvents has been studied by a variety of methods in several laboratories. Only pertinent references concerning adenine and thymine (or uracil) association are cited here (Hamlin *et al.*, 1965; Kyogoku *et al.*, 1966, 1967a,b, 1969; Katz and Penman, 1966; Katz, 1969; Nagel and Hanlon, 1972). Most of these studies were done in nonpolar organic solvents in order to minimize solvent competition for hydrogen-bonding sites and to eliminate hydrophobic stacking interactions. The reason for such studies is to try to understand the origin of the specificity in the basic pairing scheme in polynucleotide complexes in terms of "electronic complementarity" (Kyogoku *et al.*, 1969).

In the present study, we were able to examine dimer–dimer association in chloroform for the first time because of the availability of alkyl phosphotriesters. With suitable blocking groups on the sugar hydroxyls, these compounds are readily soluble in chloroform. The two residues of the dimers were observed to behave almost independently in the association process; hence, there is little restriction of rotation about the bonds in the backbone. The relationship of these findings to recent studies on unperturbed dimensions of polynucleotides in aqueous solution is discussed.

Materials

1-Cyclohexylthymine (T) and 9-ethyladenine (A) were purchased from Cyclo Chemical Co. Deuteriochloroform (99.8%) from Merck and Co. was dried over type 4A molecular sieves before use. All compounds used for hydrogen bonding were dried for 3 hr in an Aberhalden with refluxing acetone in the presence of P_2O_5 on aspirator vacuum.

Chemical Synthesis of the Phosphotriesters

General Strategy. The protected thymidine triester, Tp(Et)T, was prepared by condensation of a protected nucleoside (MTrT) with the ethyl ester of the protected nucleoside 5′-phosphate (d-EtpTOAc) followed by purification by silica gel chromatography. The preparation of the protected adenine triester, dAp(Et)dA, involved first synthesis of the fully protected triester d-DMTrA^{Bz}p(C_2H_5)A$^{(Bz)_2}$OBz. The *N*-benzoyl groups were then selectively removed by treatment with hydrazine hydrate (Letsinger *et al.*, 1968).

Analytical Procedures. Thin layer chromatography was carried out on Eastman 6060 chromatogram sheets. Paper chromatography was performed *via* the descending technique on Whatman 3MM paper using the following solvent sys-

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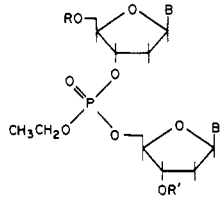
tems: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent C, 1 M ammonium acetate–95% ethanol (3:7, v/v, pH 7.5). Ultraviolet spectra were recorded on a Cary-15 uv spectrophotometer. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by Micro Tech Laboratories, Skokie, Ill.

Preparation of Starting Materials. 5′-*O*-Mono-*p*-methoxytritylthymidine, N^6-benzoyl-5′-*O*-di-*p*-methoxytrityldeoxyadenosine (Schaller *et al.*, 1963), 3′-*O*-acetylthymidine 5′-phosphate (Khorana and Vizsolyi, 1961), and $N^1,N^6,O^{3'}$-tribenzoyldeoxyadenosine 5′-phosphate (Ralph and Khorana, 1961) were prepared by methods described in the literature.

2,4,6-Trimethylbenzenesulfonyl chloride (Aldrich Chemical Co.) was recrystallized from pentane before use (mp 54–55°). Anhydrous pyridine was prepared by distillation of commercial material containing *p*-toluenesulfonyl chloride onto potassium hydroxide, followed by redistillation onto molecular sieves (Linde Type 4A).

Ethyl Ester of 5′-Mono-p-methoxytritylthymidylyl-(3′–5′)-3′-O-acetylthymidine (*Structure I*). The ethyl ester of 3′-*O*-



I. MTrTp(Et)TOAc or Tp(Et)T
R = MMTr, R' = acetyl, B = thymine

II. d-DMTrAp(Et)AOBz or dAp(Et)dA
R = DMTr, R' = benzoyl, B = adenine

acetylthymidine 5′-phosphate was prepared by the reaction of pyridinium thymidine 5′-phosphate (900 mg; 2.5 mmol) with 100 ml of anhydrous ethanol in a solution containing 35 ml of anhydrous pyridine, 64 ml of triethylamine, and 10 g of dicyclohexylcarbodiimide. After 3 days at room temperature 20 ml of water was added, the solution was filtered, the solvents were evaporated, and the residue was dissolved in 150 ml of water. The aqueous mixture was extracted with ether (2 × 100 ml), followed by concentration and examination by paper chromatography and paper electrophoresis. One compound, R_F[c] 0.84 (R_F[c](dpTOAc) 0.66), R_m 0.50 relative to dpT, was observed corresponding to the desired d-EtpTOAc. The aqueous solution was evaporated and the residue was dissolved in pyridine.

5′-*O*-Mono-*p*-methoxytritylthymidine (514 mg; 1 mmol) was added to the pyridine solution containing d-EtpTOAc (2.5 mmol) and the solvents were evaporated. The resulting gum was rendered anhydrous by repeated evaporation with pyridine (3 × 5 ml). The gum was dissolved in 4 ml of dry pyridine and treated with mesitylenesulfonyl chloride (550 mg; 2.5 mmol) for 24 hr at room temperature. Additional pyridine (4 ml) was added, and the solution was treated with 8 ml of ice water. The aqueous solution was taken up in 40 ml of chloroform. The chloroform solution was extracted with water (2 × 40 ml) and dried over anhydrous sodium sulfate. After filtration the chloroform solution was concentrated and chromatographed on a silica gel column (3 × 30 cm) using ethyl acetate (1 l.) and 50% ethyl acetate–tetrahydrofuran (750 ml) as solvents. Material with R_F^{EtOAc} 0.16 (silica gel) was collected, the solvents were evaporated, and the residue was precipitated from tetrahydrofuran by addition of hexane. The triester was further purified by thick-layer silica gel chromatography using ethyl acetate as solvent to give 82 mg of MTrTp(C_2H_5)TOAc: R_F^{EtOAc} 0.15, $R_F^{H_4furan}$ 0.65 (silica gel tlc); mp 117–120°.

A portion of the triester (2 mg) was treated with 0.2 ml of 80% acetic acid for 4 hr at room temperature. The solvent was evaporated and the residue was treated with 1 N sodium hydroxide in 0.2 ml of 50% aqueous pyridine for 10 min at room temperature, followed by neutralization with Dowex pyridinium resin. The resin was filtered and the solution after concentration was subjected to silica gel tlc. One nucleotidic compound was observed corresponding to previously prepared unprotected Tp(C_2H_5)T, $R_F^{H_4furan}$ 0.33 (Miller *et al.*, 1971).

Ethyl Ester of 5′-O-Di-p-methoxytrityldeoxyadenylyl-(3′–5′)-3′-O-benzoyldeoxyadenosine (*Structure II*). N^6-Benzoyl-5′-*O*-di-*p*-methoxytrityldeoxyadenosine (673 mg; 1 mmol) and the bispyridinium salt of $N^1,N^6,O^{3'}$-tribenzoyldeoxyadenosine 5′-phosphate (445 mg; 0.55 mmol) were dried by evaporation with anhydrous pyridine (3 × 5 ml). The gum was dissolved in dry pyridine (5 ml) and treated with mesitylenesulfonyl chloride (327 mg; 1.5 mmol) at 0°. After 4 hr at room temperature, the solution was treated with ice water (5 ml). Additional pyridine (10 ml) and water (150 ml) were added, followed by extraction with *n*-butyl alcohol (75 ml). The butanol phase contained d-DMTrA^{Bz}, $R_F^{10\%\ MeOH-H_4furan}$ 0.64, and d-DMTrA^{Bz} pA$^{(Bz)_2}$OBz, $R_F^{10\%\ MeOH-H_4furan}$ 0.22 (silica gel tlc). Removal of the protecting groups by treatment with 50% concentrated ammonium hydroxide in pyridine (1 ml, 3 days) and with 80% aqueous acetic acid (1 ml, 15 min) gave, after paper chromatography, dA, R_F^A 0.63, and d-ApA, R_F^A 0.33. The butanol was evaporated and the residue was dried by evaporation with pyridine (3 × 5 ml). The gum was dissolved in a solution containing dry *N*,*N*-dimethylformamide (10 ml), dry 2,6-lutidine (5 ml), and dry ethanol (5 ml), and was treated with *p*-toluenesulfonyl chloride (1.9 g; 10 mmol) at 0°. After 1 hr at room temperature, the solution was cooled to 0°, additional ethanol (5 ml) and *p*-toluenesulfonyl chloride (1.9 g) were added, and the solution was kept another hour at room temperature. Ice water (10 ml) was added, the solvents were evaporated, and the residue was evaporated with several portions of ethanol. The residue was dissolved in chloroform (50 ml) and extracted with water (2 × 50 ml). The combined aqueous extracts were extracted with chloroform (50 ml), and the combined chloroform extracts were dried over anhydrous sodium sulfate. After filtration, the chloroform was evaporated and the residue was chromatographed on silica gel (3 × 35 cm) using ethyl acetate (500 ml) and tetrahydrofuran (250 ml) as solvents. Material with R_F^{EtOAc} 0.25 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from tetrahydrofuran by addition of hexane to give d-DMTrA^{Bz}p(C_2H_5)A$^{(Bz)_2}$OBz: mp 118–122°; yield 294 mg (40%); uv λ_{max}^{EtOH} 235, 277 mμ; λ_{min}^{EtOH} 223, 265 mμ.

Anal. Calcd for $C_{71}H_{63}N_{10}O_4P \cdot H_2O$: C, 64.14; H, 4.92; N, 10.53. Found: C, 64.01; H 4.63; N, 10.52.

The d-DMTrA^{Bz}p(C_2H_5)A$^{(Bz)_2}$OBz (290 mg; 0.21 mmol) was dissolved in a solution containing pyridine (4 ml), glacial acetic acid (1 ml), and 85% hydrazine hydrate (0.16 ml). The solution was kept at room temperature for 16 hr, after which the solvents were evaporated and the residue was evaporated with several portions of ethanol. The resulting pale yellow syrup was dissolved in chloroform (50 ml), extrated with water (50 ml), and, after concentration, chro-

matographed on silica gel (3 × 25 cm) using ethyl acetate (500 ml), tetrahydrofuran–ethyl acetate (1:1, 500 ml), 5% methanol in tetrahydrofuran (500 ml), and 10% methanol in tetrahydrofuran (250 ml) as solvents. Material with $R_F^{\text{EtOAc-H}_4\text{furan, 1:1}}$ 0.04 was collected, the solvents were evaporated, and the residue was precipitated from tetrahydrofuran by addition of hexane to give d-DMTrAp(C_2H_5)-AOBz: mp 115–119°; yield 156 mg (69%); uv $\lambda_{\max}^{\text{EtOH}}$ 235, 258 mμ; $\lambda_{\min}^{\text{EtOH}}$ 222, 247 mμ.

Anal. Calcd for $C_{50}H_{51}N_{10}O_{11}P \cdot H_2O$: C, 59.04; H, 5.25; N, 13.77. Found: C, 59.05; H, 5.06; N, 13.88.

A solution containing d-DMTrAp(C_2H_5)AOBz (5 mg) in pyridine (0.25 ml) was treated with 2 N sodium hydroxide (0.25 ml) at room temperature for 10 min. The solution was neutralized with Dowex 50-X pyridinium resin and, after filtration and evaporation, the residue was treated with 80% acetic acid (0.5 ml) for 1 hr at room temperature. The solvents were evaporated and the residue was subjected to paper chromatography. The only nucleotidic material observed corresponded to unprotected d-Ap(C_2H_5)A, R_F^C 0.57 (Miller *et al.*, 1971).

Methods

Infrared Measurements. All infrared (ir) measurements were made on a Beckman IR-9 spectrophotometer. Spectra were recorded in the absorbance mode using double beam operation without compensation for the solvent. The wave number range from 3100 to 3800 cm^{-1} was normally examined. A fixed slit width calculated to give a resolution of 2 cm^{-1} was routinely used. A sodium chloride cell (1.09-mm pathlength) and an infrasil quartz cell (5.00-mm pathlength) were employed.

Analysis of the Infrared Data. Association constants and ir extinction coefficients were determined from the dependence of the ir spectra on the concentrations of the interacting molecules. Concentrations were calculated from dry weights of compounds put in solution. Ultraviolet extinction coefficients determined from such solutions were sometimes used in subsequent concentration determinations. Data were analyzed graphically using several linear relations which are easily derived from the Beer–Lambert law and the mass-action expression. For self-association, the following equation applies (Kyogoku *et al.*, 1967a)

$$A_{\bar{\nu}} = \frac{\epsilon^2 l^2}{2K}\left(\frac{C_0}{A_{\bar{\nu}}} - \frac{1}{\epsilon l}\right) \tag{1}$$

Here $A_{\bar{\nu}}$ is absorbance of a band at wave number $\bar{\nu}$ due to unassociated molecules, ϵ is the molar extinction coefficient, l is the pathlength, K is the self-association constant, and C_0 is the input monomer concentration. In a typical self-association experiment, spectra were recorded at several input concentrations in the range 0.005–0.08 M in a 1.09-mm cell. Plots of $A_{\bar{\nu}}$ *vs.* $C_0/A_{\bar{\nu}}$ yielded straight lines in accordance with eq 1, and K and ϵ were calculated from the slope and the intercept on the axis of abscissas. Spectra of 0.005 M solutions were also taken in a 5-mm cell for verification of band positions and extinction coefficients.

For cross-association, equimolar solutions were prepared in the concentration range 0.002–0.02 M, and spectra were read in a 5-mm cell. Absorption bands of both associated and unassociated species were analyzed. The bands due to the unassociated species were analyzed according to eq 2, where

$$A_{\bar{\nu}} = \frac{\epsilon^2 l^2}{K}\left(\frac{C_0}{A_{\bar{\nu}}} - \frac{1}{\epsilon l}\right) \tag{2}$$

C_0 is the input concentration of each interacting species. Strictly speaking, this equation should be corrected for self-association (Kyogoku *et al.*, 1967a), but this relatively small correction involves rather laborious calculations which were not considered worthwhile in view of the rather low accuracy of the ir measurements. The absorption bands due solely to bonded species were analyzed by eq 3. A plot of $(A_{\bar{\nu}})^{1/2}$ *vs.*

$$(A_{\bar{\nu}})^{1/2} = \epsilon l\left(\frac{C_0}{(A_{\bar{\nu}})^{1/2}} - \frac{1}{(\epsilon l K)^{1/2}}\right) \tag{3}$$

$C_0/(A_{\bar{\nu}})^{1/2}$ should be linear, and again ϵ and K can be calculated from the slope and the intercept on the $(C_0/(A_{\bar{\nu}})^{1/2})$ axis. A similar plot for self-association has been used by Pitha (1970).

In the case of adenine self-association, there exists the possibility of chain formation; *i.e.*, the two bonded adenine molecules are further complexed with other adenines through the remaining bonding sites. The self-association data for A and dAp(Et)dA were therefore also analyzed in a way which allows for chain formation. If one assumes the same association constant for each successive step in the association, then the equilibrium can be represented by

$$M_{n-1} + M_1 \rightleftharpoons M_n$$

$$K = \frac{m_n}{m_{n-1}m_1} \tag{4}$$

where m_n is the molar concentration of the n-mer. It follows that

$$m_n = Km_1m_{n-1} = K^2m_1{}^2m_{n-2} = \ldots = K^{n-1}m_1{}^n \tag{5}$$

From conservation of mass

$$C_0 = \sum_{n=1}^{\infty} nm_n = \sum_{n=1}^{\infty} nK^{n-1}m_1{}^n \tag{6}$$

It can be shown that

$$\sum_{n=1}^{\infty} nK^{n-1}m_1{}^n = \frac{m_1}{(1 - Km_1)^2} \tag{7}$$

and, hence

$$m_1 = C_0(1 - Km_1)^2 \tag{8}$$

With the use of the Beer–Lambert law, this equation can be rearranged to give

$$A_{\bar{\nu}} = \frac{(\epsilon l)^{1/2}}{K}\left((\epsilon l)^{1/2} - (A_{\bar{\nu}}/C_0)^{1/2}\right) \tag{9}$$

In this case, $A_{\bar{\nu}}$ *vs.* $(A_{\bar{\nu}}/C_0)^{1/2}$ yields a linear plot the slope and intercept of which can be used to calculate K and ϵ.

The values of ultraviolet extinction coefficients used in the analysis were as follows: 1-cyclohexylthymine, 1.08×10^4 at 274 nm; Tp(Et)T, 1.08×10^4 at 266 nm; 9-ethyladenine,

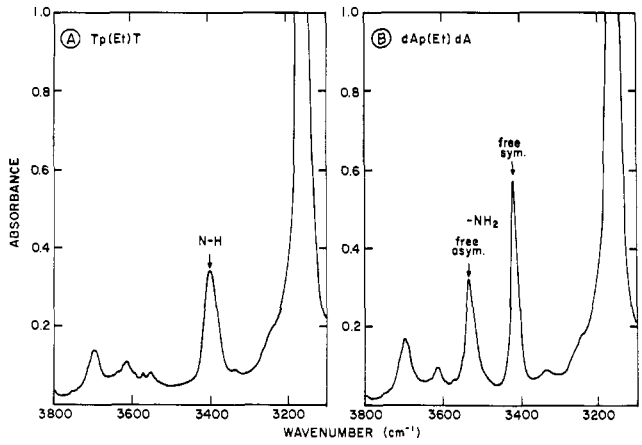


FIGURE 1: Infrared spectra of (A) Tp(Et)T at 0.004 M residue concentration; (B) dAp(Et)dA at 0.005 M. In deuteriochloroform solution with a 5-mm infrasil cell; room temperature.

1.3×10^4 at 260 nm; dAp(Et)dA, 1.39×10^4 at 258 nm. These values are per base residue and have an accuracy of about $\pm 5\%$.

Results

The self-association and cross-association of adenine and thymine in chloroform were studied at the monomer–monomer, monomer–dimer, and dimer–dimer levels. Although the associations of T and A in chloroform have been examined before, these investigations have been repeated for comparison with our dimer association experiments. The results on the monomers also serve as a check on the reproducibility of the procedure. The neutral dimers used in the present study, the alkyl phosphotriesters of dinucleoside monophosphates, have an ethyl substituent on the phosphate (structures I and II). In addition, the 3′- and 5′-hydroxyls of the deoxyriboses of these neutral dimers are also substituted with blocking groups which allow them to dissolve more easily in deuteriochloroform. The thymine triester I carrying a 5′-monomethoxytrityl group and a 3′-acetyl group will be designated Tp(Et)T. The adenine triester II, which is the 5′-dimethoxytrityl 3′-benzoyl derivative, will be called dAp(Et)dA. Preparations of both Tp(Et)T and dAp(Et)dA contain a mixture of two diastereoisomers, owing to the asymmetry of the alkyl substitution on the phosphate (Miller *et al.*, 1971). While these diastereoisomers are separately identifiable in the pmr analyses of these samples, their properties in the present ir study cannot be distinguished; therefore, the results reported below on Tp(Et)T and dAp(Et)dA are due to an average of these two diastereoisomers.

Figure 1 shows ir spectra of Tp(Et)T and dAp(Et)dA at low concentration in deuteriochloroform. They are very similar to the spectra of T and A. The spectrum of Tp(Et)T in Figure 1A shows a peak at 3397 cm^{-1} due to the N–H stretch of unbonded thymine. The big peak below 3200 cm^{-1} in both spectra is due to $CDCl_3$. In Figure 1B, the unbonded $-NH_2$ groups of dAp(Et)dA give rise to two peaks at 3530 and 3417 cm^{-1} due to asymmetric and symmetric stretching, respectively. The two smaller bands above 3600 cm^{-1} result from a trace of water in the solutions. Special care was taken to keep solutions very dry, so that these "water peaks" were usually small.

At higher concentrations when self-association and cross-association occur, the absorptions due to free N–H and $-NH_2$ groups increase more slowly with increasing concentration than predicted by Beer's law. New "bonded" peaks arise at lower wave numbers. Figure 2 shows an ir spectrum of an equimolar mixture of Tp(Et)T and dAp(Et)dA having a residue concentration of 0.01 M in each. The pattern of the absorption bands is essentially the same as observed with A and T monomers. The assignments indicated in the figure are made in accordance with those of Kyogoku *et al.* (1967a). Considerable cross-association of the dimers has occurred at this concentration. The peaks due to free –NH and the symmetric stretch of free $-NH_2$ are not resolved in the mixture, but give a single, broad peak. The bands used in the calculation of association constants and extinction coefficients were the 3397-cm^{-1} band for self-association of T, the 3530- and 3417-cm^{-1} bands for self-association of A, and the 3530- and 3487-cm^{-1} bands for cross-association. The 3487-cm^{-1} band is due to stretching of the free hydrogen in a hydrogen-bonded $-NH_2$ group.

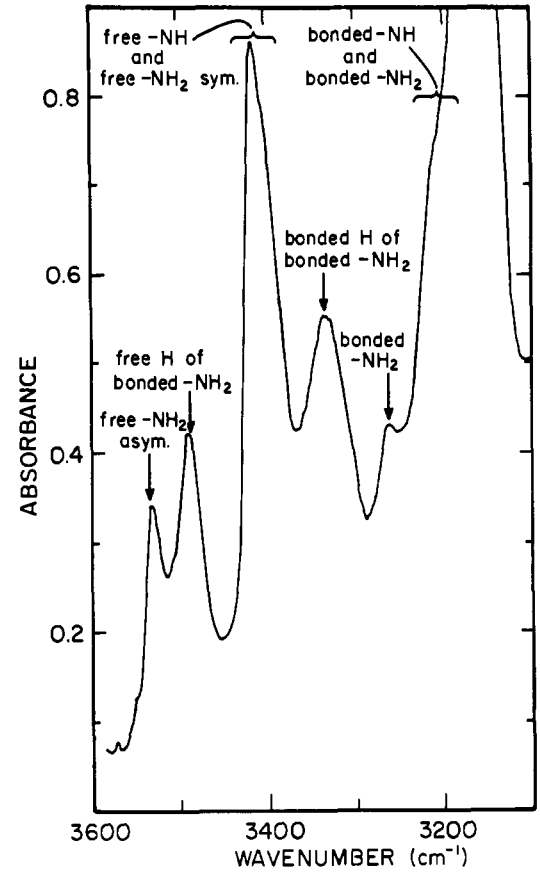


FIGURE 2: Infrared spectrum of an equimolar mixture of dAp(Et)dA and Tp(Et)T in deuteriochloroform, with a 5-mm infrasil cell; concentration of each component was 0.01 M; room temperature.

A few words of explanation are in order concerning the estimation of background absorptions to be subtracted from the uncorrected ir readings. The two bands used in calculating cross-association constants are not well resolved and, in addition, they are somewhat distorted by the broader peaks at lower wave numbers. This distortion of background, which becomes worse with increasing concentration or greater fractional association, is the greatest source of error in these experiments. The results of two methods were averaged to yield our best estimates of the constants. In the first method, a background spectrum of plain $CDCl_3$ was taken independently in the same cell. Background readings from this spectrum

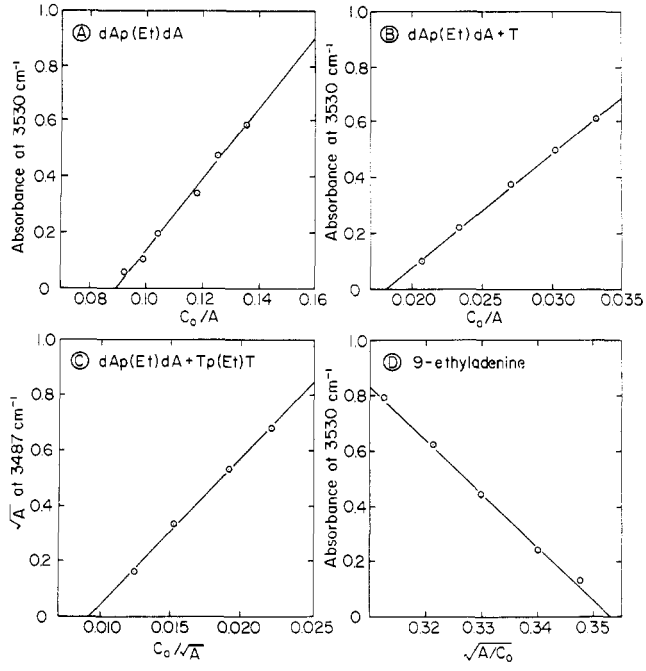


FIGURE 3: Examples of plots used to analyze association data: (A) self-association of dAp(Et)dA; (B) cross-association of dAp(Et)dA and T; (C) cross-association of dAp(Et)dA and Tp(Et)T; (D) self-association of 9-ethyladenine.

were clearly underestimates of the "correct" background. In the second approach, a straight line was arbitrarily drawn between the spectral minima above 3530 cm^{-1} and below 3487 cm^{-1}. Background readings from this line were overestimates. Our reported association constants represent an average of these two methods and are considered to have an accuracy of ±25%. Since a consistent approach was used in all the calculations, their relative accuracy for comparative purposes should be better.

Figures 3A–D show sample plots of the experimental data analyzed according to eq 1, 2, 3, and 9, respectively.

TABLE I: Infrared Extinction Coefficients (M^{-1} cm^{-1}).[a]

	Molar Extinction Coefficient			
Compd	3397-cm^{-1} Free –NH Stretch	3417-cm^{-1} Free $-NH_2$ Symmetric Stretch	3530-cm^{-1} Free $-NH_2$ Asymmetric Stretch	3487-cm^{-1} Free H of Bonded $-NH_2$
T	156			
Tp(Et)T	140			
A		223 (200[b])	116 (104[b])	
+ T				154
+ Tp(Et)T				110
dAp(Et)dA		228	108	
+ T				133
+ Tp(Et)T				104

[a] Accuracy is about ±10%. Values are per base residue. [b] Published values from Kyogoku *et al.* (1967a).

TABLE II: Intrinsic Association Constants in $CDCl_3$ at Room Temperature.[a]

Compound	K_i (M^{-1})		Lit. Values
	Self-Association		
T		3.6	3.2[b]
Tp(Et)T		4.2	
A	Pair formation	3.1	3.1[c]
	Chain formation	2.2	1.4,[d] 4.8[d]
dAp(Et)dA	Pair formation	6.5	
	Chain formation	4.0	
	Cross-Association		
T + A		92	130[b]
Tp(Et)T + A		113	
T + dAp(Et)dA		72	
Tp(Et)T + dAp(Et)dA		192	

[a] 22–24°. [b] Kyogoku *et al.* (1967b). Constants at 25°. [c] Kyogoku *et al.* (1967a). Value at 24°. [d] Nagel and Hanlon (1972). The first value is for pair formation and the second for higher associations, all taking place in a chain formation reaction.

The data in Figure 3A are for self-association of dAp(Et)dA as measured by the 3530-cm^{-1} peak. Figure 3B shows data for cross-association of dAp(Et)dA and T, again at 3530 cm^{-1}. In Figure 3C, the bonded peak at 3487 cm^{-1} was used to analyze cross-association of dAp(Et)dA and Tp(Et)T. Finally, in part D, self-association of A was analyzed with allowance for chain formation.

The numerical results of all the calculations are summarized in Tables I and II. Table I shows the molar extinction coefficients determined for the bands used in the analysis. The extinction coefficients of the free $-NH_2$ bands of A agree fairly well with published values. Also the values obtained for unbonded dimers agree closely with the values for monomers.

In Table II, the association constants are listed and compared with published values whenever available. These are all intrinsic association constants calculated in terms of residue concentration, as will be discussed later. The association constants for the monomers are in reasonable agreement with those published earlier. The intrinsic constant for cross-association of the dimers, *i.e.*, Tp(Et)T and dAp(Et)dA, is only about twice as large as that for the monomers, *i.e.*, T and A. The significance of this finding will be discussed further in the following section.

Discussion

The mathematical treatment of the spectral data on the dimer–dimer association becomes relatively simple in two extreme cases: the completely uncooperative case and the completely cooperative case. If the association is completely uncooperative, then the two residues in a dimer behave essentially in an independent manner, and the problem reduces to a monomer–monomer case. If residue concentrations are used in the mass-action expression, an intrinsic association constant (K_i) is obtained. The constants tabulated in Table II were calculated on this basis as intrinsic values. To calculate actual molecular association constants, we need to multiply such values by a statistical factor of two for monomer–dimer

association and a factor of four for dimer–dimer association. On the other hand, if the association is completely cooperative, than the only kind of associated species formed are those which have both residues hydrogen bonded. When the dimer concentration is used in the mass action expression, the calculation yields the actual association constant, whereas when the residue concentration is used, the calculation gives a value equal to one-half the association constant. If the association is only partially cooperative, then, after the two dimers are bonded together at one end, the remaining residues in these two dimers are more likely to bind to each other than to residues of other dimers. Thus, the various association processes have different entropic considerations, and the formation of each of the many possible species has its own bias factor. In this situation, the mathematics becomes intractable without additional qualification from more experimental findings.

How should the dimer–dimer association constant compare with the monomer–monomer value in each of the cases just considered? In the uncooperative case, dimer–dimer association is adequately described by an intrinsic association constant which should be approximately equal to the monomer–monomer value. In the completely cooperative case, the free energy of association in the dimer–dimer case should be about double the free energy of monomer–monomer association, and, consequently, the dimer–dimer association constant should be of the order of the square of the monomer–monomer value.

Comparison of the association constants in Table II reveals that the dimer–dimer associations in chloroform-*d* are essentially uncooperative. This conclusion is most clearly demonstrated in the situation of the cross-association. The association constant value (Table II) for 9-ethyladenine and 1-cyclohexylthymine is 92 M^{-1}. The intrinsic association constants for the two monomer–dimer cases are the same as the monomer–monomer value within experimental error. The experimental intrinsic constant of dimer–dimer association is only about twice as large (192 M^{-1}) as that for monomer–monomer association. In the cooperative case, the molecular association constant is obtained by multiplying the intrinsic value by a factor of two, *i.e.*, 384 M^{-1}. However, if the dimer–dimer association really were completely cooperative in nature, we should expect an association constant with the magnitude of the square of the intrinsic value of the monomer–monomer association, *i.e.*, a value of $(92)^2 = 8500$. Such a large association constant is clearly not observed. As for the self-association, the values of the intrinsic constants are much smaller (Table II). Therefore, the square of some of these values may not be greatly different from the products of these values multipled by a factor of two. However, wherever such a distinction can be made, such as the comparison between the intrinsic constant for T + T *vs.* the intrinsic constant of Tp(Et)T + Tp(Et)T, the self-association data are consistent with the interpretation that there is nearly a complete lack of cooperativity in the self-association process.

The absence of cooperativity in the association of the dAp(Et)dA and Tp(Et)T dimers in chloroform implies that the linkage between residues in these dimers must be very flexible in this solvent. Since the two bases in a dimer behave almost independently, there must be very little restriction of rotation about the bonds of the backbone. The above conclusion concerning the great flexibility of the backbone of the deoxydinucleoside monophosphate ethyl phosphotriesters in chloroform is pertinent to a series of recent studies about the rigidity of the backbone of polynucleotides in aqueous solution. Sasisekharan and Lakshminarayanan (1969), Lakshminarayanan and Sasisekharan (1969, 1970), Sundaralingam (1969), Arnott and Hukins (1969), and Arnott (1970), on the basis of X-ray diffraction analyses of fibers and crystals, have concluded that the possible conformation of nucleic acid backbone is severely restricted. They noted that each of the torsional angles of the backbone tends to lie within certain limits. Felsenfeld and coworkers (Eisenberg and Felsenfeld, 1967; Inners and Felsenfeld, 1970; Achter and Felsenfeld, 1971) have studied the viscosities, sedimentation velocities, light scattering, and phase separation of poly(A), poly(U), and apurinic acid under a variety of conditions, such as temperature, salt and polymer concentrations, molecular weight series, etc. The unperturbed dimensions of these polynucleotides were obtained under "ideal" θ solvent conditions, and were found to have the characteristics of random coils. However, the characteristic ratio $C_n = \langle r^2 \rangle_0 / n\bar{l}^2$, where $\langle r^2 \rangle_0$ is the mean-square end-to-end length for the unperturbed chain comprising n skeletal bonds and $\bar{l}^2$ is the mean-square bond length, was found to be unexpectedly large for these polynucleotides, being in the range of 15 ± 3, even in the absence of base-stacking effects. For polyphosphate, C_n was found to be 6.6–7.2 (Strauss and Wineman, 1958; Strauss and Ander, 1962); for L-polypeptide in the coil state, C_n was about 20 (Olson and Flory, 1972a). Inners and Felsenfeld (1970) first noted that although a certain amount of restriction can be expected to arise from steric hindrance, the steric hindrance alone does not appear to be sufficient to account for this large value of C_n. They suggested that electrostatic interaction between the charged oxygen atoms of neighboring phosphate groups may account for the high C_n values if the "effective dielectric constant" has a value of ten or less.

An extensive theoretical analysis of the spatial configuration of polynucleotide chains has recently been undertaken by Olson and Flory (1972a–c). Again they concluded that steric interference alone cannot account for the large polymer dimensions observed experimentally. Large values of C_n can be obtained only by taking account of electrostatic interactions between charged atoms in the nucleotide residue in addition to the relevant torsional and van der Waals energies. These interactions greatly reduce the rotational freedom by introducing a strong bias in favor of those conformations that minimize the coulombic energy. As a consequence of these effects, the unperturbed dimensions are increased markedly over those computed on the basis of steric interactions. A value of 3.0 was used in their calculation for the "effective dielectric constant" in the polynucleotide medium. It is also possible that interactions between the charged groups in the backbone and the solvent may introduce restrictions to rotation about the bonds of the backbone. This possibility has been alluded to by Flory (1953), but he assumed that such effects would be small.

In our present experiments, the dimers contain only *one* phosphate group, the charge of which has been neutralized by the formation of a triester. Therefore, both effects of charged phosphate–charged phosphate interaction and of charged phosphate–water interaction are *absent*. Under this condition, the backbone of the dinucleoside monophosphate ethyl phosphotriester appears to have little restriction on the rotation of its bonds, and the two base residues behave as if they are totally independent. This observation reinforces the conclusion that the restriction of the rotation of the backbone of the polynucleotide in solution cannot be due to steric hindrance alone; the data support the suggestion that the elec-

trostatic interaction between the charged groups of the polynucleotide backbone and that between the charged groups and water could be the cause of the rigidity of the polynucleotides in a random coil conformation observed in aqueous solution. Hopefully, a future study on uncharged polynucleotide alkyl phosphotriesters in aqueous solution may provide additional information to this question.

Our ir experiments give no information on the configurations (Hoogsteen type, Watson–Crick type, etc.) of the hydrogen-bonded base pairs. More specific information about which atoms are involved in hydrogen bonding can be obtained from proton magnetic resonance. For 1-cyclohexyluracil and 9-ethyladenine association, it has been suggested that both the normal Watson–Crick configuration and Hoogsteen pairing exist to about the same extent (Katz, 1969). In the uncooperative association of dimers, there is also a possibility of forming chains involving bridging of two dimers by another dimer. This kind of association may be related to our observation of two –NH resonances separated by about 1 ppm in the pmr spectrum of an equimolar mixture of dAp(Et)dA and Tp(Et)T (Alderfer, DeBoer, and Ts'o, 1972[1]). Further pmr studies will hopefully elucidate these details of the association. This problem is also amenable to molecular weight measurements of the mixed species in solution.

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